

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Visser et al.

Examiner: W. Baker

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For: METHODS FOR PRODUCING AND
TRANSFORMING CASSAVERE
PROTOPLASTS

Assistant Commissioner for Patents
Washington, DC 20231

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DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, Richard Visser, declare and say:

1. Protoplasts of certain species have been found to regenerate into plants. However, very specific conditions were found to be necessary for such regeneration. These specific conditions are unpredictable. Also, protoplasts obtained only from certain parts of particular plants may be capable of regeneration. It is not predictable which part of an individual plant would provide protoplasts that would have such a capability. Moreover, the stage of development of the plant tissue from which a protoplast is obtained also determines if a protoplast is capable of regeneration. Protoplasts have different degrees of differentiation in different stages of plant tissue development. The potential of a protoplast to develop into a mature plant may be dependent upon the stage of differentiation of the protoplasts. In what stage of differentiation a protoplast needs to be for a particular plant, and from what part of a particular plant such a protoplast can be obtained is unpredictable.

Protoplasts obtained from different sources would have disparate characteristics. Thus, knowledge of the regenerative capabilities of protoplasts obtained from one source would provide little, if any, information regarding the regenerative capabilities of protoplasts obtained from another source. For example, the regenerative capabilities of protoplasts

obtained from expanded leaves of a certain plant would not provide substantial information regarding the regenerative capabilities of protoplasts obtained from embryogenic suspension cultures of the same plant.

2. Regeneration of plant protoplasts can follow two distinctly different developmental pathways: somatic embryogenesis and adventitious shoot formation. Somatic embryogenesis leads to the formation of somatic embryos which, as in the case with zygotic embryos, possess a shoot and a root meristem. Under appropriate conditions, these somatic embryos develop into complete plants with mature roots. Adventitious shoot formation leads to shoots which do not possess a root meristem; that is, a complete plant is not formed. These shoot formations may be termed "plantlets." What pathway is followed by particular protoplasts is determined by the factors outlined above.

3. Cassava is very recalcitrant for plant regeneration of its protoplasts. Until the present invention, attempts to produce plants from protoplasts taken from various parts of the cassava plant have not been successful.

There is only one report of shoot regeneration from protoplasts of cassava (Shahin and Shephard, 1980). In the method of Shahin et al., protoplasts were isolated from well expanded leaves. These protoplasts developed under specific conditions into green calli. Some of these green calli developed shoots, however at a very low frequency. An example of such a shoot is shown in Fig. 1e of Shahin et al.

The shoots obtained by Shahin et al. are not equal to plants. Plants by definition should be complete. That is, complete plants contain developed shoot and root systems. The shoots shown in Fig 1e do not have roots. Shahin et al. state that they tried to induce root formation on these shoots; however, they did not report that they were successful.

Moreover, Shahin et al. described their shoot formation as not efficient. In particular, Shahin et al. state that they have "not yet fully defined the conditions for efficient shoot formation." (Shahin et al., page 464, last paragraph.) Usually root formation occurs after shoots have been obtained.

Furthermore, cassava is grown commercially for its thickened roots. The shoots shown by Shahin et al. accordingly have no commercial value.

4. Despite considerable efforts, the disclosure of Shahin et al. concerning production of "plants" (or merely shoots) from protoplasts of cassava has been found to be non-reproducible. That is, plant regeneration from protoplasts (isolated from leaves, stems, and roots) has never been repeated (Anonymous, 1985; Nzoghe, 1991; Anthony et al., 1995; Sofiari, 1996). Embryogenic cells found in the apical meristems, young leaves or somatic embryos cultured on auxin supplemented media were used in attempts for regeneration. (Stamp and Henshaw, 1987a; Raemakers et al., 1993a). However, protoplasts isolated from these tissues gave in the best case green callus and adventitious roots (Sofiari, 1996).

The references listed below evince that the work of Shahin et al. was not reproducible. Some of the references accompany this Declaration in Exhibit A, as indicated, with relevant sections highlighted. The other references are available upon request.

Schöpke et al., (1993) "Plant Protoplasts and genetic Engineering" *Biotechnology in Agriculture and Forestry* 23: 273-275. (See Exhibit A.)

Konan et al. (1997) "An Efficient Mass Propagation System for Caaava (*Manihot esculenta* Crantz) Based on Nodal Explants and Axillary Bud-Derived Meristem" *Plant Cell Reports* 16:444-449. (See Exhibit A.)

McDonnell SL, Gray VM, (1997). "Transformation and culture of cassava protoplasts." *African Journal of root and tuber crops*; 2:169-172. (protoplast from leaves of *in-vitro* plants; no plants);

Cabral GB, Aragao FJL, Monte-Neshich DC, Rech EL, (1993) "Transient gene expression in cassava protoplasts." *First international meeting of the cassava biotechnology network*, CIAT working document 123:422-246. (protoplast from leaves of *in-vitro* plants; no plants);

CIAT (1986) "Annual report cassava program" 1985; *Centro Internacional de Agricultura Tropical, Cali, Columbia*, pp. 334-360. (protoplast from leaves of *in-vitro* plants; no plants);

Anthony/Davey/Power/Lowe 1995. "An improved protocol for culture of cassava protoplasts." *Plant cell tissue organ culture* 42:299-302, copy enclosed. (See in particular p. 299: "The recalcitrancy of cassava as an active dividing protoplasts system *in vitro* is well documented (Byrne 1984), and there is to date, only one non-reproducible report on plant regeneration from cassava protoplasts (Shahin et

al 1980)." (Thus, protoplast from leaves of *in vitro* plants; no plants) (See Exhibit A.)

Anonymous 1985, CIAT annual report pp. 197-217 (no regeneration of plants)

5. Taylor et al. describe obtaining *plantlets* from FEC. They do not describe complete plants. Root and shoot "poles" refer to very early stages of root and shoot development.

6. Embryogenic suspension cultures (ESC) substantially differ from FEC. The plant tissue in FEC and ESC are in different stages of development. FEC are in a pre-embryogenic stage of development. That is, FEC do not contain integrated embryo structures. In contrast, ESC contain integrated embryogenic structures, including early embryos, i.e., heart and torpedo-shaped embryos, to secondary and mature embryos.

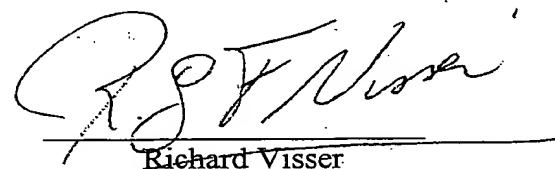
Sofiari (1996) used embryogenic suspension initiated as described by Raemakers (1993) for the isolation of protoplasts. Although a large number of variables and treatments, amongst which also the procedure described by Shahin et al. (1980), were tested, this did not result in the formation of plants.

7. Surprisingly, only protoplasts obtained from FEC, as set forth by the present invention, were capable of regenerating into complete mature plants.

In accordance with the present invention, protoplasts are isolated from FEC and cassava plant regeneration is accomplished via the developmental pathway of somatic embryogenesis. The plants which are obtained are complete; that is, they possess shoots and roots. (This is evinced by the PhD thesis of Sofiari (1996), referred to in the specification.)

8. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. Further that these statements were made with the knowledge that willfully false statements, and the like, made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willfully false statements may jeopardize the validity of the application of any patent issued thereon.

Date: October 3, 2002



A handwritten signature in black ink, appearing to read "R. Visser", is written over a horizontal line. Below the line, the name "Richard Visser" is printed in a smaller, standard font.

Declaration Under 37 C.F.R. 1.132 - 294-52 (3).DOC